Initial segment Kv2.2 channels mediate a slow delayed rectifier and maintain high frequency action potential firing in MNTB neurons.

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Abstract

The medial nucleus of the trapezoid body (MNTB) is specialized for high frequency firing by expression of Kv3 channels which minimize action potential (AP) duration and Kv1 channels, which suppress multiple AP firing during each calyceal giant EPSC. However the outward K⁺ current in MNTB neurones is dominated by another unidentified delayed rectifier. It has slow kinetics and a peak conductance of ~37nS; it is half-activated at -9.2±2.1mV and half-inactivated at -35.9±1.5mV. It is blocked by several non-specific potassium channel antagonists; including quinine (100mM) and high concentrations of extracellular tetraethylammonium (TEA; IC₅₀ = 11.8mM), but no specific antagonists were found. These characteristics are similar to recombinant Kv2-mediated currents. Quantitative rt-PCR showed that Kv2.2 mRNA was much more prevalent than Kv2.1. A Kv2.2 antibody showed specific staining and Western blots confirmed that it recognized a protein ~110Kd which was absent in brainstem tissue from a Kv2.2 knockout mouse. Confocal imaging showed that Kv2.2 was highly expressed in MNTB neurone axon initial segments. In the absence of a specific antagonist, Hodgkin-Huxley modeling of voltage-gated conductances showed that Kv2.2 has a minor role during single APs (due to its slow activation) but assists recovery of Nav channels from inactivation by hyperpolarizing inter-spike potentials during repetitive AP firing. Current clamp recordings during high frequency firing and characterization of voltage-gated Na⁺ channel inactivation confirmed this hypothesis. We conclude that Kv2.2 containing channels have a distinctive initial segment location and crucial function in maintaining AP amplitude by regulating the inter-spike potential during high frequency firing.
Introduction

Potassium currents have multiple and diverse roles in shaping electrical signaling, with different suites of voltage-gated and rectifying non-gated channels setting neuronal membrane potentials, firing threshold, action potential waveform and firing patterns. Identification of the channel family and subunits contributing to these functions in native neurones is complicated by their heterogenous subunit composition, their particular functional localization to the plasma membrane and by the absence of specific antagonists for some families. For these reasons the full complement of the K^+ channels and subunits underlying native delayed rectifying K^+ current is still not known for any identified central neurone. We have focused on studying the potassium currents of a ‘simple’ neurone within the medial nucleus of the trapezoid body (MNTB) which serves as a relay in the binaural circuits involved in sound source localization.

These neurones receive the glutamatergic calyx of Held giant synapse, which reliably triggers postsynaptic APs with large, well timed EPSCs which have a magnitude of around 30 times firing threshold. The in vivo calyceal input fires spontaneously at frequencies between 0-100Hz (Kopp-Scheinpflug et al., 2003) and to evoked stimuli at up to 800 Hz for short periods without AP failure (Taschenberger & von Gersdorff, 2000). Although the inter-spike membrane potential is elevated during stimulus trains, each EPSP triggers only one postsynaptic AP. Voltage-gated K^+ channels shape AP waveform and firing frequency in the presynaptic terminal and postsynaptic MNTB neurone. Postsynaptic Kv1 channels suppress AP firing during the EPSP decay so that the output of the MNTB neurone precisely follows the timing and pattern of the presynaptic incoming AP train (Brew & Forsythe, 1995; Dodson et al., 2002; Brew et al., 2003; Gittelman & Tempel, 2006). Kv3 channels speed AP repolarization, promoting short APs and high frequency firing (Wang & Kaczmarek, 1998; Rudy et al., 1999;
A-type potassium currents (I_A) mediated by Kv4 channels are also present in mouse MNTB neurones (Johnston et al., In Press).

Under voltage-clamp, the greater part of the MNTB outward current is resistant to Kv1 and Kv3 antagonists, indicating that another K^+ current must also play a role in MNTB neurone excitability. In this paper we use biophysical, immunohistochemical, molecular and modeling methods to characterize this voltage-gated K^+ conductance. It is a slow-gating delayed rectifier that generates large currents on depolarization and is mediated by Kv2.2-containing channels. The slow kinetics of this Kv2 conductance limits its contribution to repolarization of a single AP. However during high frequency repetitive stimulation, cumulative activation of this conductance causes hyperpolarization of the inter-spike potential, promoting recovery of voltage-gated Na^+ channels (I_{Na}) from inactivation and so enhancing AP firing.

**Materials and Methods**

*Brain slice preparation.* Brain slices were prepared as described previously (Barnes-Davies & Forsythe, 1995; Wong et al., 2006). Briefly, CBA/Ca mice aged P10-P19 were decapitated in accordance with the UK animals (Scientific Procedures) Act 1986 and the brain was removed in a slush of iced artificial CSF (aCSF) of composition (in mM) 250 sucrose, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 0.5 ascorbic acid, 26 NaHCO_3, 4 MgCl_2, 0.1 CaCl_2, gassed with 95% O_2/5% CO_2 (pH7.4). The brain was placed ventral side up and the brainstem removed with a cut at the level of the pons and angled ~20° toward the cerebellum. The rostral surface was then mounted on the stage of an Integraslice 7550 MM (Campden Instruments, Loughborough, UK). Four to five transverse slices 120-150µm thick were cut from the area containing the MNTB. Slices were then transferred to aCSF containing (in mM): 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4,
2 Na-pyruvate, 3 myo-inositol, 0.5 ascorbic acid, 26 NaHCO₃, 2.5 MgCl₂, 0.5 CaCl₂, gassed with 95% O₂/5% CO₂ (pH7.4), incubated at 37°C for 1 hour and then stored at room temperature until required.

**Electrophysiology.** For recording, one slice was transferred to a Peltier controlled environmental chamber on the stage of an upright E600FN microscope (Nikon, Tokoyo, Japan) and continuously perfused with aCSF (~1ml min⁻¹ at 25-27°C, or 35-37°C for current clamp and Nav recordings). Patch pipettes were pulled from thick walled borosilicate glass (GC150F-7.5, Harvard Apparatus, UK) and whole-cell patch recordings were made from visually identified neurones in the MNTB using an Optopatch amplifier (Cairn, Faversham UK).

For studying K⁺ currents the pipette solution contained (in mM): 97.5 K-Gluconate, 32.5 KCl, 10 HEPES, 5 EGTA (or 10 BAPTA) 0.01 ZD 7288 (to block Iₜₜ) and 1 MgCl₂ (pH 7.2 with KOH) and had series resistances of 4-12MΩ. The perfusing aCSF contained 1µM tetrodotoxin (TTX) to block Iₙa, and usually contained 3mM TEA and 10nM DTx-I unless otherwise stated. For current clamp recording; pipette solutions were identical but with ZD 7288 omitted and the perfusing aCSF contained no ion channel blockers.

For studying Na⁺ currents the pipette solution contained (in mM): 120 CsCl, 10 TEA-Cl, 5 4-aminopyridine, 5 EGTA, 10 HEPES, 5 NaCl (pH 7.2 with NaOH) and had series resistances of 3-8 MΩ. The perfusing aCSF contained 20µM DNQX, 10µM bicuculline, 1µM strychnine, 100µM CdCl₂, 3mM TEA, 1mM CsCl. Leao et al (2005) demonstrated that, when recording Nav currents from MNTB slices, space clamp and voltage error only induced minor inaccuracy, however we blocked around half of the sodium current by including 5nM TTX in the aCSF (IC₅₀ of the MNTB subunits Nav1.1 & 1.6 is ~5nM, Catterall et al., 2003) to further reduce voltage clamp error.
The average series resistance from 28 recordings was 6 \pm 0.4M\Omega. All series resistances were compensated by at least 75% with 10\mu s lag. The holding potential in voltage-clamp was –77mV; current clamped neurones were held at ~–70mV. Data were excluded from analysis if more than 250pA was required to hold at ~−70 mV.

Data acquisition and analysis. Data were acquired using pClamp 9.2 and a Digidata 1322A interface (Molecular Devices) filtered and digitized at 2 kHz and 10-50kHz respectively for K\(^+\) currents. For Na\(^+\) currents data was filtered at 5kHz and digitised at 50kHz.

For K\(^+\) currents: no leak subtraction was performed on the K\(^+\) currents. K\(^+\)-mediated leak is nonlinear (outwardly rectifying) as described by the GHK current equation; conventional leak subtraction (which is linear) fails to subtract all the leak. Therefore the residual current in figure 2D, when fit by a GHK current equation of the form

\[ I = \frac{Pz^2}{1-e^{-(zFV/RT)}} \]  

(Hille, 2001) is consistent with complete block of the voltage-activated current components. For example, the DTx-I sensitive current (shown in Supplemental Figure S1) 40 ms into each pulse was corrected for non-linearity of the single channel current using a modified GHK equation of the form

\[ i = \alpha \cdot \left( \frac{F^2V}{RT} \right) \frac{([K^+]_{in} - [K^+])_{out}}{1-e^{-(FV/RT)}} \]  

where terms have their usual meaning and \( \alpha \) is a normalization factor (Clay, 2000). For estimating the IC\(_{50}\) for TEA block of the Kv2 current, concentrations of 3, 10, 30 and 100mM were all measured relative to 1mM TEA (which was already present to block Kv3 currents). A Hill equation of the form: Proportion of channels occupied (P\(_{occ}\)) = [TEA] / IC\(_{50}\) + [TEA] was then fit to the data, with the P\(_{occ}\) value for 1mM TEA varied to give the best fit (which gave a P\(_{occ}\) of 0.05 for 1mM). Junction potentials of –7 mV have been added to stated voltages for the K\(^+\) based pipette solution. For Na\(^+\) currents: voltages were corrected for –4 mV junction
potentials and leak subtraction was performed using the scaled pulse method to minimize capacitive artifacts.

Activation and inactivation parameters were determined by a Boltzmann function of the form $I = I_{\text{max}} / (1 + e^{(V-V_{1/2})/k})$ with variables $I_{\text{max}}$, $V_{1/2}$ and $k$ (the slope factor). All fits were performed using Clampfit 9.2 (Molecular Devices) or Excel (Microsoft) with least squares minimization. Data is presented as mean ±SEM and either a two-tailed Student’s t-test (paired or unpaired) or an ANOVA was used to assess significance, which is indicated by * for 95% confidence and ** for 99%.

**Quantitative rt-PCR (Qrt-PCR):** RNA extraction (each homogenate consisted of MNTBs pooled from 8 mice) was carried out using RNeasy lipid tissue mini kit (Qiagen) and samples were DNAse treated (DNAfree, Ambion). RT–PCR was performed on 1–2µg of the RNA sample with SuperScript III first strand synthesis kit (Invitrogen) using the oligo-dT primers. The resulting cDNA was then used for fluorescence PCR (Power SYBR Green PCR Master Mix, Applied Biosystems and ABI PRISM 7000 thermal cycler). Primer sequences were: Kv2.1 forward TGGAGAAGCCCAACTCATC, reverse CAATGGTGGAGAGGACAATG; Kv2.2 forward CACCTGGCTTGAACAGAAAG, reverse TTGCTTCCGATAATGTCCAC; Kv3.1 forward TGAACAAGACCGAAATCGAG, reverse CGAAGGTGAACCAGACAC; actin forward TGCTCCTCCTGAGCGCAAGTACTC reverse CGGACTCATCGTACTCCTGCTTG. Amplification occurred under the following conditions: 50°C 2min, 95°C 10min, followed by 95°C 15s and 60°C 60s for 40 cycles. Primer efficiency was >95% assed by serial dilutions and construction of a standard curve. Relative expression levels were determined using the comparative $C_T$ method (User Bulletin 2, ABI PRISM Sequence Detection System, pp. 11–15, 1997, PE Applied Biosystems).
**Immunohistochemistry:** P13 CBA mice brainstems were frozen in Tissue Tek (Sakura) using hexane and dry ice. Cryostat sections (20µm) were mounted on polylysine-coated slides and fixed in 4% paraformaldehyde/PBS for 25min at 4°C. After washing 3x5 minutes in 100mM PBS with 0.1% Triton X-100 (PBS-T) the slides were treated with citrate buffer (pH6.0) for 20min at 95°C (antigen retrieval). After rewashing in PBS-T, sections were blocked with 1% BSA, 1% goat serum in PBS-T for 1h at 20°C and then incubated with the primary anti-Kv2.2 (Alomone, 1:1000) and in some cases also anti-Kv1.2 (Neuromab, 1:1000) in blocking buffer overnight at 4°C. After washing with PBS-T, the secondary antibody (goat anti-rabbit Alexa-fluor 488, 1:1000, and for anti-Kv1.2 goat anti-mouse Alexa-flour 568, 1:500, Molecular Probes) was applied for 2hr at 20°C, then mounted with Vectashield (Vector Labs). DAPI was included to visualize cell nuclei. Images were obtained using a Zeiss LSM 510 Meta confocal microscope. Control sections underwent identical procedures, but were pre-incubated with the immunizing peptide. Fluorescence intensity was measured in grey levels using ImageJ 1.38 (NIH, USA)

**Solutions and drugs:** All chemicals were obtained from Sigma except: TTX (Latoxan), dendrotoxin-I and r-stromatoxin-1 (Alomone), linopiridine, DNQX, ZD7288 and bicuculline (Tocris Cookson) and E4031 (Calbiochem).

**Immunobloting.** Proteins were extracted from a Kv2.2 KO (Lexicon, TF1551) and WT animals brain tissues using manual homogeniser and NP-40 lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.0 mM EDTA, 1% NP-40) freshly supplemented with phosphatase and protease inhibitors, followed by brief sonication and centrifugation. Samples (40 mg of proteins) were diluted into SDS-PAGE sample buffer and resolved by
SDS-PAGE. Immunoblotting was performed using anti-Kv2.2 primary antibody (Alomone, Jerusalem, Israel) and chemiluminescence detection (ECL, GE Healthcare, UK).

**Neuron Modeling.** A Hodgkin-Huxley type model of the Kv2.2 conductance of the form:

$$ I = P * \text{GHK}(v) * m * h $$  \hspace{1cm} (equation 1)

was implemented using the NEURON simulation software (Hines & Carnevale). $P$ dictated the magnitude of the conductance and was adjusted to give a current magnitude equal to the measured values, $\text{GHK}(v)$ specified the Goldman-Hodgkin-Katz dependence on voltage, $m$ was the activation variable and $h$ was the inactivation variable. The steady-state values of $m$ and $h$ were given by the Boltzmann functions in Figure 2E. Time constants (in ms) for changes in $m$ were given were derived from least square fits to the measured activation and deactivation time constants (Model figure A) and were specified as

$$ \text{Taum} = \frac{1003}{0.0307\exp((v+95.2)/11.0)+99.0\exp((v+39.7)/-10.9)} + 4.19 $$  \hspace{1cm} (equation 2)

Time constants for changes in $h$ were fixed at 900 ms, independent of voltage.

The single-compartment MNTB model described by Macica et al. (2003) was manipulated using the NEURON simulation software (Hines & Carnevale 2001). Conductances for the different channels incorporated in this model are given in Figure 5B. The reversal potential of the leak current was set to -70 mV. Synaptic currents were constructed using a reversal potential of 0 mV and were of the form:

$$ I = B * \text{Ga} * (v - 0) + C * \text{Gb} * (v-0) $$  \hspace{1cm} (equation 3)

Where $v$ was voltage, $G$ was conductance and $B$ (and $C$) were given by,
X = z \times (\exp(-t/0.75) - \exp(-t/0.33)) \quad (X = B \text{ or } C) \quad (equation 4)

Where z was a scaling factor set to normalise the maximum of X to 1.

**Results**

Under voltage-clamp the majority of outward current in MNTB neurones is resistant to block by perfusion of Kv3 and Kv1 channel antagonists, 3mM TEA and 10nM DTx-I, respectively (Figure 1). The current-voltage (I/V) relationship for outward current recorded under control conditions (Fig 1A) and for that measured after antagonist perfusion (Fig 1B) were measured at a latency of 40 ms (dashed vertical line) and the result plotted in figure 1C. The recordings were made from animals aged P10-P14 days. The TEA and DTx-I-insensitive current has two distinct components, as shown in Figure 1B: a fast activating transient A-type current (arrow) which has been characterized previously (Johnston et al., In Press) and a slowly activating delayed rectifier.

**Figure 1 - Near Here**

**The slowly activating delayed rectifier:** Isolation of a slowly activating outward current was achieved by pharmacological block of the Kv1 and Kv3 components and inactivation of the A-current by a prepulse to -37 mV for 20 ms (Figure 2A). Increasing step depolarizations generated a slowly activating and deactivating current (see figure 2A & 2F). The rising phase of the current was well fit by a single exponential, with a time constant which varied with voltage (Figure 2B). Activation rates were slow at negative voltages ($\tau = 39.6 \pm 5.2$ms at -27mV, n=6) and accelerated e-fold with 17.7mV depolarization to asymptote at ~2.3ms by +43mV. A Boltzmann function fit to tail currents (Figure 2C) gave a half-activation, $V_{1/2ac}$ of $-9.2\pm2.1$mV and $k_{ac}$ of $-9.0\pm0.4$
(Figure 2E, diamonds, n=6), indicating that the current activates from ~−30mV and is maximally activated by +20mV.

Steady-state inactivation was assessed using the protocol in Figure 2D and fitting the current evoked at +43mV with a Boltzmann function. This gave a $V_{1/2in}$ of $-35.9±1.5$mV and $k_{in}$ of $10.8±0.8$ (Figure 2E, triangles, n=7) and this current exhibits little steady-state inactivation at resting potentials. Following activation, the conductance slowly inactivated (Figure 2D) but neither single nor double exponentials gave reliable fits over a range of voltages. The fitting problem is most likely caused by changes in voltage error associated with the declining current passing across the residual series resistance as the current inactivates, therefore quantification was limited to measuring the time to half-inactivation, which at -7mV (where complete inactivation occurs) was 669±32ms (n=5).

Following a train of brief depolarizing pulses, the rate of inactivation was similar to that during a sustained depolarization (data not shown) which is consistent with the absence of ‘U’-type inactivation (Klemic et al., 1998). The slow deactivation of this tail current (Figure 2F) also exhibits voltage-dependence. The current was maximally activated by stepping to +53mV for 15ms (>5τ) prior to stepping back to test voltages. Decays were fit with single exponentials and mean time constants are plotted in Figure 2G, showing an e-fold increase with 30.6mV hyperpolarization.

Figure 2 - Near Here

Which $K^+$ channel families do not contribute to this slow delayed rectifier?

We have made use of well characterized pharmacological tools to identify Kv1 and Kv3 conductances in MNTB neurones, but many other pharmacological agents were tested in order to distinguish between the voltage-gated families which might underlie the slow delayed rectifier. As above, the Kv1 subfamily was eliminated with DTx-I (Dodson et al.,
10nM was a maximally effective dose because 100nM had no further effect). No DTX-I-insensitive low-voltage-activated currents were observed in the MNTB and potential contributions from Kv1.3 and Kv1.4 channels are unlikely since CP339 818 (Nguyen et al., 1996; Jager et al., 1998) had no effect on mouse MNTB currents (5-10 µM n=3, data not shown) as previously observed in the rat (Dodson et al., 2002). We eliminated the Kv3 family by perfusion of 1-3mM TEA (Brew & Forsythe, 1995; Wang & Kaczmarek, 1998; Coetzee et al., 1999; Song et al., 2005) which at these concentrations also blocks BKCa. There is already evidence against the presence of large calcium-sensitive K+ currents in the MNTB (Brew & Forsythe, 1995); nevertheless, we conducted voltage-clamp experiments with low external calcium ([Ca2+]/[Mg2+]o = 0.5:2.5 mM) and with high concentrations of internal calcium buffer (5 mM EGTA or 10 mM BAPTA) so this delayed rectifier is not a BK or SK current. Although many of the Kv7 channels (KCNQ/M-current) mediate slowly-activating currents, they are all sensitive to low concentrations of linopirdine (Robbins, 2001; Lawrence et al., 2006; Vervaeke et al., 2006; Hu et al., 2007) and the slow outward current was unaffected by 50µM linopirdine (n=2, data not shown). The Kv4 family underlies A-type currents (Birnbaum et al., 2004) and Kv4.3 is responsible for the A-current observed here (Johnston et al., In Press) but A-currents were removed by voltage-dependent inactivation (see Figure 2A). EAG/ERG/ELK (Kv10, 11 & 12) subfamilies were excluded from the present study as addition of E4031 (1µM) to the internal patch solution left the current unaffected (Gessner & Heinemann, 2003; Royer et al., 2005; Lamarca et al., 2006; Furlan et al., 2007). Contributions from HCN channels were routinely blocked by inclusion of ZD7288 (10 mM) in the patch solution. We could find no evidence for significant activation of KNa channels in the MNTB under these physiological recording conditions. We were unable to induce significantly increased outward currents on adding 23 mM [Na+] to internal solutions. Furthermore, addition of
2mM [ATP], and reduction of [Cl]₀ to 7mM (n=3, data not shown) had no influence on the measured outward currents. Taken together these results exclude all of the known K⁺ channel families (Coetzee et al., 1999): Kv1, Kv3, Kv4, EAG, KCNQ, BK, KNa, SK, HCN, with the exception of Kv2.

**Pharmacological agents acting on the slow delayed rectifier.** This putative Kv2 conductance was largely insensitive to 3 mM [Ba²⁺]₀ (n=5, data not shown), but perfusion of 100 mM quinine caused complete block of the voltage-gated component. The remaining current was fit by the GHK current equation (Figure 3A), consistent with mediation by leak channels (see methods). Unfortunately, quinine is not specific and will block a range of K⁺ channel families and voltage-gated Na⁺ channels (data not shown) so it cannot be used to assess physiological significance in current clamp recordings. One report suggests that a purified tarantula toxin called stromatoxin-1 modifies the gating of Kv2 channels and has a high affinity for Kv2.1 subunits (Escoubas et al., 2002). Recent reports using a recombinant-stromatoxin-1 in neocortical pyramidal neurones confirmed that it reversibly blocks native Kv2.1 currents but with a much reduced affinity (Guan et al., 2007). We tested r-stromatoxin-1 (Alomone) using concentrations in the range of 100nM-5µM (n=5, three different lots, data not shown) but it had no effect on the slow delayed rectifier.

**Figure 3 - Near Here.**

The classic K⁺ channel antagonist 4-aminopyridine (4-AP) is non-specific, blocking Kv1, Kv2, Kv3 and Kv4 currents (Coetzee et al., 1999) as well as this slow delayed rectifier (Figure 3B). Although low concentrations of external TEA (1-3 mM) are relatively specific for Kv3 channels, higher concentrations will also block many K⁺
channels. The MNTB slow delayed rectifier was sensitive to high concentrations of TEA, showing a dose response curve with an IC\textsubscript{50} of 11.8 mM (Figure 3C). The current was insensitive to low concentrations of TEA applied internally (2 mM, n=2 data not shown), which points to an incompatibility with Kv2.1 subunits (Taglialatela et al., 1991). The pharmacological profile from studies of recombinant Kv2 channels in expression systems exhibit greater sensitivity to TEA and 4-AP than observed here, but the pharmacology can be influenced by incorporation of subunits from the “electrically silent” families (Post et al., 1996; Robertson, 1997) and since both Kv5.1 and Kv6.4 are expressed in the MNTB (Lein et al., 2007) it is possible that the native Kv2 channel is not a Kv2.2 homomultimer.

We performed Qrt-PCR on tissue dissected from MNTB nuclei to determine which Kv2 subunits are expressed. Figure 4A shows that Kv2.2 transcripts were present at considerably higher levels than Kv2.1 (n=3). This is consistent with the above pharmacology, the lack of ‘U’-type inactivation and absence of immunostaining for Kv2.1 in the mouse MNTB (REW Fyffe, personal communication).

**Figure 4 - Near Here**

The specificity of an anti-Kv2.2 antibody was verified by Western blot (Figure 4B); the antibody stained a single band at the appropriate molecular weight for mouse Kv2.2 (Accession number XP_984002) that was absent in the presence of the blocking (immunising) peptide (Figure 4D, n=3) and not detected in brains from homozygous Kv2.2 knockout mice. We found that anti-Kv2.2 broadly labeled most neurones in the auditory brainstem, with neurones in the MNTB and the ventral nucleus of the trapezoid body (VNTB) showing particularly strong focal labeling (Figures 4C, 4D, n=5) and some labeling of blood vessels was observed. Similar staining was observed in rat MNTB.
(n=2, data not shown). In addition to the somatic labeling of the MNTB, which was predominantly cytoplasmic, intensely stained spots and rod-like structures were observed of up to 10 mm in length. Figure 4D shows a higher magnification image of one MNTB nucleus. These high intensity areas are clearly seen in a line-plot of the fluorescence intensity measured across the medio-lateral axis of the MNTB (dashed white line and plotted as grey scale level above blocking peptide control). Two distinct levels of staining above background were seen, an intermediate level in the MNTB neuronal somata (20-50au, stars) and the very bright spots and rods (above 100au, indicated by red dashed lines). The bright regions were always peripheral to cytoplasmic staining and often adjacent to somatic profiles.

**Kv2.2 channels are concentrated in the axon initial segment**

Confocal imaging showed that these intensely labeled regions were associated with individual MNTB neurones (Figure 4E, arrow). We observed diffuse Kv2.2 immunostaining in the cytoplasm of principal MNTB neurones (consistent with protein synthesis, trafficking or presence in other internal organelles). The most intense labeling was observed in processes either immediately adjacent or near to principal neurone somata; the 4 mm thick confocal projection in Figure 4E shows a single MNTB neurone with green cytoplasmic staining of Kv2.2, a blue DAPI stained nucleus and an intense green band which appears to form part of the axon initial segment (AIS). To test this hypothesis we used co-immunolocalization with Kv1.2 antibodies known to be present in MNTB AIS (Dodson et al., 2002), and elsewhere in the brain. The co-labeling with Kv1.2 (red) and Kv2.2 (green) shows clear association of both Kv1 and Kv2 K+ channels with the initial part of the MNTB neurone axon (Figure 4F). This is consistent with evidence of interaction between the measured Kv1 and Kv2 currents, as indicated by the non-parallel and overlapping I/V curves in control and following DTx-I application (see
supplementary Figure S1 A&B). DTx-I blocks Kv1 current at negative voltages but the outward current at positive voltages (mediated by Kv2 channels) increases in amplitude and occasionally surpasses control levels (supplemental figure S1 A Black and purple arrows respectively); implying that the (unblocked) Kv1 conductance reduces voltage-clamp quality for positive voltage steps, consistent with location of Kv1 and Kv2 channels either in the same or succeeding electrotonic compartments. In support of this conclusion Kv2 current was not observed in any excised outside-out patches from MNTB somata (n=8) but was present in all whole-cell recordings (n>67). These electrophysiological observations, combined with the Kv2.2 immunostaining are consistent with the hypothesis that initial segment Kv2.2 channels mediate the somatic slow delayed rectifier current.

**Modeling of the Kv2.2 conductance.**

In the absence of a specific antagonist to Kv2.2 we investigated the physiological function of this slow delayed rectifier by using a Hodgkin-Huxley type model of an MNTB neurone. The model was based on the published MNTB neurone model of Macica et al., (2003) containing: Nav, K_LVA (Kv1) K_HVA (Kv3) and I_{leak} (Figure 5B) to which a Kv2.2 conductance was added. The A-current was not included in the model for simplicity, as it has been shown to have minimal effects in trains of action potentials (Johnston et al., In Press). APs were elicited by suprathreshold ‘calyceal synaptic’ inputs (Figure 5C) in the presence or absence of the model Kv2.2 conductance. At low firing frequencies (50 Hz) the presence of the Kv2.2 current (shown in Figure 5 C&D) had little effect. The inter-spike interval was slightly depolarized and AP amplitudes slightly reduced when the Kv2.2 was excluded from the model. However at higher AP firing rates the Kv2.2 current did not completely deactivate during the shorter inter-spike interval, enabling accumulation of the current during the early stages of a 200 Hz train (Figure 5D, right).
The elevated Kv2.2 conductance during the inter-spike interval opposed the summing synaptic conductances, resulting in a more hyperpolarized inter-spike potential (compare with arrow marking inter-spike interval in the absence of the kv2.2 current; Figure 5C, right). In the absence of this hyperpolarizing drive from the Kv2.2 current, the AP amplitudes were reduced (Grey arrow, Figure 5C) and this attenuation was due to increased Nav inactivation with the more depolarized inter-spike intervals.

**Figure 5 – Near here**

**Hyperpolarization of the inter-spike potential.**

The rapidly gating high voltage-activated Kv3 current is known to make a major contribution to action potential repolarization in MNTB neurones and other fast spiking cells (Rudy et al., 1999). So the slow activation of Kv2 channels means that Kv2 conductance is unlikely to contribute to single APs when AP half-widths are around 0.5ms, which is consistent with the model. Both Kv1 and Kv3 currents turn off relatively rapidly (Brew & Forsythe, 1995) with their activity essentially following the AP waveform (Klug & Trussell, 2006), and there is little evidence for their cumulative activation during repetitive stimulation until the highest frequencies are reached (e.g. 600Hz; (Klug & Trussell, 2006). The model shows that activation of Kv2.2 conductance can accumulate at high firing frequencies (200Hz and above) and hyperpolarizes the inter-spike potential. To test this prediction in native MNTB neurones we evoked trains of 60 APs under current clamp conditions using direct current injection of simulated synaptic currents. This method of stimulation avoids variability caused by quantal release and short-term depression during repetitive stimulation. The AP waveforms are similar to those evoked by orthodromic synaptic stimulation (Dodson et al., 2003; Postlethwaite et al., 2007) and are more physiological than step current injection.
The inter-spike potential was measured under current-clamp recording conditions during a 50Hz and 200Hz stimulus trains. The experimental responses are shown in Figure 6A (50Hz black, 200Hz grey traces). At 50Hz the inter-spike potential showed a small decline of 1.9±0.5mV by the 60th spike (compare dashed line vs arrow in figure 6A black trace, n=7, p=0.014). In contrast the inter-spike potential during a 200Hz train in the same cell (Figure 6A, grey trace) declined by 4.7±0.7mV (Figure 6C, n=4, p=0.0075). Similar results were seen in two further cells, but these were excluded from averaged data because the first stimulus generated 2 APs; multiple firing of MNTB neurones is a common finding in mice (Brew et al., 2003). This cumulative and frequency dependent hyperpolarization was also seen in 4 cells patched with 10mM BAPTA in place of EGTA in the pipette, ruling out any affects of Ca^{2+}-activated K^{+} channels. Furthermore, no ATP was included in the patch pipette ruling out any effects from the Na/K ATPase. Note that the fast after-hyperpolarization (AHP) of MNTB neurones is mediated by Kv3 channels, whose activation follows the AP waveform (Klug & Trussell, 2006). The frequency-dependent decrease in inter-spike potential confirms the predicted effect of Kv2.2 from the MNTB model (Figure 5 C & D).

**Sensitivity of the Nav current to the inter-spike potential**

The Kv2-MNTB model also predicted effects on the amount of Nav channels available to activate during a train. However, the measured inter-spike hyperpolarization during a high frequency AP train is only a few millivolts. We therefore examined the MNTB Na^{+} current inactivation in greater detail. The steady-state inactivation of the Nav current was measured under voltage clamp as shown in Figure 7A (inset) with the peak Na^{+} current...
evoked on voltage steps to -4mV plotted against the pre-pulse conditioning voltage. The curve was fit with a Boltzmann function giving a $V_{1/2in}$ of -55.4 ±1.7mV and a slope ($k_{in}$) of 6.3 ±0.1 (Figure 7A, n=5). This data confirms that MNTB Nav channels exhibit little steady-state inactivation at resting membrane potentials; however during repetitive stimulation the inter-spike potential occurs over the steepest part of the inactivation voltage range. Therefore a K$^+$ current need only induce small changes in the inter-spike potential to have a pronounced effect on the amount of available Nav channels through out a train.

**Figure 7 – Near Here**

To further demonstrate the influence of the inter-spike potential on Nav channel inactivation we used a voltage clamp protocol designed to maximally activate the $I_{Na}$ in a repetitive fashion (Figure 7B, $V_{Test}$). Each test pulse was followed by a 5ms “inter-spike period” reproducing the inter-spike interval for a 200Hz AP train (see the protocol in Figure 7B with an AP overlaid in red). A 1ms ‘repolarizing’ step to -67mV ($V_{AHP}$, Figure 7B) mimicked the AHP mediated by fast Kv3 currents, then the remaining 4ms inter-spike voltage ($V_{is}$) was stepped over a range of potentials from -55mv to ±-5mV (Figure 7B), matching the observed range of the inter-spike potential during AP trains.

The first $I_{Na}$ in each train was the largest, since there was little resting inactivation at these voltages, with the second depolarizing step following the 5 ms inter-spike period being reduced by 40.5 ±5.8% (n=7, p=<0.001). This incomplete recovery from inactivation was a constant and measurements were made relative to this second pulse in the train. A train of 60 repeats showed a 17±14.4% increase in $I_{Na}$ (n=7, p=0.004 measured at the 60th spike, compared to the 2nd) when $V_{is}$ was matched to the measured values during an action potential train (voltages in Figure 6C were used).
Sodium current magnitude stabilized around the 15th repetition (Figure 7C & D, black). In contrast, when $V_{ls}$ was maintained constant at $-55$ mV, $I_{Na}$ decreased by $25.1 \pm 8.6\%$ (n=8, p=0.02, measured at the 60th $V_{test}$) and was still declining by the 60th repeat (Figure 7 C&D, blue trace).

The model showed that Kv2 channels caused a more hyperpolarized inter-spike potential (Figure 5 D). So to test what effect the absence of Kv2.2 would have on the sodium current's inactivation we set $V_{ls}$ to depolarize, giving mild summation of the inter-spike potential (Fig 7C, final $V_{ls}$=50.6mV). Under these conditions we observed a 49.9 ±4.0% decline in $I_{Na}$ from the 2nd $V_{test}$ (Figure 7C&D, green trace, n=5, p=0.007). These results further validate the predictions of the MNTB model, suggesting that Kv2 channels function to hyperpolarize the inter-spike potential during high frequency stimulation, promoting recovery of Nav channel inactivation and ultimately maintaining AP amplitude during repetitive stimulation.

**Kv2.2 current increases with development and has a tonotopic gradient**

The Kv2 current magnitude increased with postnatal age (assessed in the presence of 3mM TEA and 10nM DTx-I to isolate Kv2.2). A significant increase in current magnitude was observed across the age range of P10-14 (P<0.0001, ANOVA) and additional data from P18-P19 animals showed a further increase with the same level of significance, as shown in figure 8A. Note that absolute current amplitude is plotted; since the Kv2 channels are not uniformly distributed, normalization to cell capacitance is of little relevance. But for comparison purposes this normalization was performed on pooled data from the P10-14 animals and compared with the P18-19 group. This gave Kv2 current amplitudes of $63.16 \pm 10.77\text{pA pF}^{-1}$ (n=6) and $148.85 \pm 20.26\text{pA pF}^{-1}$ (n=5), respectively and are significantly different (P=0.003). The $V_{0.5ac}$ for Kv2.2 was unchanged with age (P18-9 vs P10-14).
Both Kv1 and Kv3 channels show a functional tonotopic distribution along the medio-lateral axis of the MNTB (Brew & Forsythe, 2005) with higher magnitude currents in the medial versus the lateral neurones. To test for a tonotopic gradient of Kv2.2 current, the MNTB was divided into 3 parts; medial, intermediate and lateral (Figure 8B). The Kv2.2 current magnitude was plotted against location for data from P12 mice. Use of data from other ages was avoided to minimize developmental variation (see Figure 8A). A significant gradient of current magnitudes was detected (P=0.03, figure 8C) with a tendency for larger current magnitude in lateral compared to medial neurones. This is opposite to the gradient for the Kv3 and Kv1 channels (Li et al., 2001; Brew & Forsythe, 2005), but similar to that of a previously reported slowly deactivating current of the rat MNTB (Brew & Forsythe, 2005).

Discussion

Kv1, Kv3 and Kv4 potassium currents have well recognized roles in maintaining high fidelity transmission and generating fast action potential waveforms in the MNTB. Here we identify and characterize the fourth voltage-gated K⁺ conductance mediating a delayed rectifier in the MNTB. Although there are no specific blockers for this current, we have studied it by conducting voltage-clamp experiments under conditions where other known conductances are blocked. Our electrophysiology, pharmacology, PCR and immunohistochemical data provide strong evidence that this slow-activating conductance is mediated by Kv2.2-containing channels located in the axon initial segment. It acts during high frequency action potential firing, contributing to
hyperpolarization of the inter-spike potential and enhancing recovery of sodium channels from inactivation.

**Voltage-gated conductances of MNTB neurones.**

It is well established that a low voltage-activated current in the MNTB is mediated by Kv1 channels, while a high voltage-activated current is mediated by Kv3 channels. In the presence of Kv1 and Kv3 blockers, more than half (6nA at +30mV) of the high voltage-activated outward current remains and we have shown that this is mediated by a Kv2 channel. The A-current mediated by Kv4 channels also makes a small contribution in mouse (Brew et al., 2003) (Johnston et al., In Press) but it is absent in rat MNTB neurones (Dodson et al., 2002). This A-current is largely inactivated at resting potentials and is completely inactivated by the voltage-protocols used in this study. Together with previous publications, these data show that mouse MNTB neurones express voltage-gated potassium channels from each of the four classic voltage-gated $K^+$ channel gene families. Their distinct locations and functions suggest general rules for the role of these gene families in regulating neuronal excitability: Kv1 regulates AP firing threshold, Kv2 assist recovery of sodium channels from inactivation, Kv3 enables fast action potential repolarization and Kv4 channels further influence AP waveform and inter-spike interval, conditional on the prior voltage history.

The high voltage-activated slow delayed rectifier is a Kv2.2 channel. Our whole cell patch data showing slow kinetics and positive voltage-activation of the antagonist-insensitive current are compatible with recombinant Kv2 channels (Coetzee et al., 1999). The general pharmacology of this current is also consistent with this interpretation: it is insensitive to the Kv1 antagonist dendrotoxin-I and is insensitive to low concentrations of TEA (1-3mM, which block Kv3 and BK channels), but is blocked by higher
concentrations showing an IC$_{50}$ of 11.8 mM TEA (see figure 3). It is also partially sensitive to 4-aminopyridine (which blocks Kv1, Kv3, Kv2 and Kv4 channels) and insensitive to E4031 an antagonist of EAG and related channels, and the Kv7/KCNQ antagonist linopirdine. It is blocked by micromolar levels of quinine (Schmalz et al., 1998). The biophysical data and pharmacological profile is consistent with the MNTB delayed rectifier being of the Kv2 family, and the immunohistochemical and molecular evidence shows that the current is mediated by channels containing Kv2.2 subunits.

Early immunostaining data reported high Kv2 expression in the cerebellum and brainstem (Hwang et al., 1993) with little subcellular overlap in expression of Kv2.1 and Kv2.2. Recent in situ hybridization studies shows no evidence of Kv2.1 and low levels of Kv2.2 mRNA in the MNTB (Lein et al., 2007). Our PCR data confirms that Kv2.2 is expressed in the MNTB while Kv2.1 mRNA levels were negligible. Immunohistochemistry confirmed broad expression of Kv2.2 in brainstem neurones, accompanied by intense immunofluorescence of MNTB neurone axon initial segments. We also demonstrate functional restriction of the slow current to this compartment (see supplemental figure S1). The axonal location of Kv2.2 is also observed in other brain areas e.g. cortical inhibitory neurones and perforant pathway axons in the dentate gyrus (Hwang et al., 1993). The Kv2.2 staining is in marked contrast to the focal Kv2.1 aggregates associated with synaptic profiles in spinal motoneurones (Muennich & Fyffe, 2004) and hippocampal neurones (Misonou et al., 2004), indeed, the mouse MNTB shows no detectable expression of Kv2.1 by immunohistochemistry (REW Fyffe, personal communication).

Other recent evidence has suggested that the MNTB nucleus expresses sodium-dependent K$^+$ channels (Yang et al., 2007). In contrast to this report our recordings
made in the absence of added [Na\(^+\)], show large sustained outward K\(^+\) currents (routinely >6 nA). We found no evidence for significant contributions from K\(_{\text{Na}}\) channels under our conditions in the MNTB: there was little change in the outward currents on adding [Na\(^+\)] to the patch solution, the outward currents were not chloride-dependent nor blocked by ATP. One difference between the studies is that in order to make substitutions of [Na\(^+\)], while maintaining the [K\(^+\)] electrochemical gradient, hyperosmotic solutions were used by Yang et al in their whole cell recordings. Our data (collected at normal osmolarity) do not require internal sodium ions to generate large voltage-gated outward K\(^+\) currents and both immunohistochemical and molecular data are consistent with the dominant slow outward K\(^+\) conductance being Kv2.2.

The presence of Kv2.2 mRNA and the intense immunolabelling in the MNTB strongly support the functional expression Kv2-containing channels, but it is unclear whether the channels are homo- or heteromeric. Heteromers of Kv2.1 and Kv2.2 subunits are unlikely due to lack of expression of Kv2.1 in the MNTB and specific evidence that these subunits do not associate in other systems (Malin & Nerbonne, 2002). It is well established that both Kv2.1 and Kv2.2 subunits form functional channels with members of the electrically silent families (i.e. Kv5, 6, 8 & 9) resulting in alterations in activation ranges and pharmacological sensitivities (Post et al., 1996; Robertson, 1997; Salinas et al., 1997). Certainly mRNA expression for ‘silent’ subunits have been detected in the MNTB, e.g. Kv5.1 and Kv6.4 (Lein et al., 2007) so co-expression with Kv2.2 is theoretically possible. It is currently unclear whether our observed insensitivity to recombinant ScTx-1 reflects subunit heterogeneity or the recombinant origin of the toxin. However, as each subunit contributes 1/4 to the selectivity filter (which is close to the binding site of 4-AP and TEA) a Kv2.2 heteromeric channel would readily explain the decreased sensitivity to these channel blockers.
A physiological role for Kv2.2 channels in the MNTB.

The slow activation rate and depolarized activation range of the Kv2.2-mediated current means that it can make little contribution to single APs in the MNTB, particularly because MNTB APs are so fast, with half-widths of less than 0.5 ms. This contrasts with other studies of Kv2 channels showing that Kv2.1 contributes to AP repolarization (Blaine & Ribera, 2001; Malin & Nerbonne, 2002). However in these areas (SCG and xenopus neurones) AP half-widths range between 2.5-3.5ms, so allowing more time for Kv2 activation during one AP. The unusual speed of the MNTB action potential is achieved by expression of Nav1.6 sodium channels (Leao et al., 2005) combined with the high expression of Kv3 potassium channels (Wang & Kaczmarek, 1998; Li et al., 2001). The AP waveform is dominated by the fast activating and deactivating Kv1 and Kv3 channels (Brew & Forsythe, 1995), their activity follows the AP waveform turning off with the falling phase of the AP (Klug & Trussell, 2006). It is in the period between spikes where the slower Kv2.2 channels are free to exert their effect. Despite the fact that only a fraction of the Kv2.2 channels turn on with a single action potential their slow deactivation allows the activity to accumulate, particularly at higher frequencies. A frequency-dependent role for Kv2 (Shab) currents has also been proposed at the drosophila neuromuscular junction (Ueda & Wu, 2006).

In the absence of specific antagonists for native Kv2.2 currents, our proposal based on the properties of voltage-gated Na⁺ currents and modeling of the Na⁺ and K⁺ currents, is that Kv2 channels contribute to maintaining high frequency firing by assisting recovery of Na⁺ channels from inactivation, thus ensuring that more Na⁺ channels are able to contribute during trains of APs.
As the inter-spike potential falls on the steepest part of the inactivation range for the sodium current, small changes in inter-spike voltage will have a large effect on the availability of Nav channels. We show that after the 1st spike in a 200Hz train around 60% of the sodium current is able to reactivate, but with the cumulative membrane hyperpolarisation this amount increased by ~17%. More importantly the available I_{Na} did not decline with spike number, which would result in eventual failure of transmission. Kv2.2 channels are open during the inter-spike potential, open K⁺ channels hyperpolarize the membrane potential. So to support the proposed role of Kv2.2 channels we simulated their absence by either depolarising the inter-spike potential or maintaining it at the initial level. In both cases we observed a significant reduction in the available Nav channels throughout a 200Hz train. Together, these findings support the conclusion that the Kv2.2 mediated hyperpolarisation will sustain firing by preventing cumulative inactivation of I_{Na} during high frequency trains. In addition, the magnitude of this Kv2.2 conductance increases with maturation, similar to many other properties of this synapse which together facilitate higher frequency transmission (Taschenberger & von Gersdorff, 2000) (Joshi & Wang, 2002; Joshi et al., 2004).

Further work is required to determine the subunit stoichiometry of Kv2 channels but heteromers with electrically silent subunits have been demonstrated in expression systems (Post et al., 1996; Robertson, 1997; Salinas et al., 1997). Such precision has yet to be achieved in native neurones, but with further knowledge of the spectrum of subunits expressed in the MNTB principal neurone, the MNTB could provide the first answer to this problem.

**Acknowledgements**

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References


Figure 1. A slow delayed rectifier in MNTB neurons

A. Control outward K⁺ currents evoked by step commands from a pre-pulse voltage of -97 mV to the indicated voltages, in 10 mV increments under voltage-clamp conditions. B. Repeat of the same voltage-clamp protocol in the same neuron following perfusion of DTx-I (10 nM) and TEA (3 mM). Arrow indicates unmasking of a small A-type current. C. The mean current-voltage (I/V) relationship for control (black, n=18) and following DTx and TEA perfusion (grey, n=18). Note that more than half of the outward current is insensitive to DTx and TEA, this current is therefore not mediated by Kv1 or Kv3 channels.

Figure 2. Kinetics of the slow high voltage-activated current

A. In the presence of TEA (3 mM) and DTx-I (10 nM) an initial depolarization from -97 to -37 mV activates, and then inactivates the A-current. After 20 ms further voltage steps were applied to activate the remaining voltage-gated current. B. The voltage-dependence of activation is fit by a single exponential; the mean time-constant is plotted against voltage and shows an e-fold increase with 17.7 mV depolarization (n=6) grey trace. C. Shows an enlargement of the dash-boxed area in part A; an activation curve was measured from these tail currents, measured at the latency indicated by the filled diamond. D. A sustained conditioning voltage step (8 seconds) over voltages ranging from -107 to +43 mV showed the isolated delayed rectifier to have slow inactivation, which was assessed by measuring the current on stepping to +43 mV (filled triangle). E. A Boltzmann function was fit to the magnitude of activation tail-currents (filled diamonds) and another Boltzmann function was fit to the inactivation data (filled triangles) from the data shown in C & D (see text for mean data). F. Deactivation rates were measured after maximally activating the current by stepping to +53mV for 15ms and then stepping to the indicated voltages. Single exponentials fit the tail currents; with the average data plotted
Figure 3. There were no specific blockers of this slow delayed rectifier. A. In the presence of TEA and DTx-I (black trace) quinine (100μM) blocked the remaining sustained voltage-gated current (grey trace). The GHK current equation fits the quinine-insensitive current showing that it is not voltage gated, but reflects the K⁺ leak currents. Inset traces show the respective control (black) and test (grey) traces from the same cell (without inactivation of the A-current, pre-pulse potential –97mV). B. 4-aminopyridine (4-AP, 5-10mM, n=4) gives a partial block of the current; inset shows control and test (grey) current traces from the same cell (pre-pulse potential –77mV). The 4-AP-insensitive current is not fit by the GHK current equation, indicating that voltage-gated current remain. C. High concentrations of TEA blocked the slow-delayed rectifier. A dose response curve to TEA was conducted and data pooled from multiple cells. Each point is the mean current from 3-4 neurons. The grey curve is a fit to the Hill equation and gives an IC₅₀ of 11.8 mM. The open triangle was a free parameter to allow for block by 1 mM TEA which was present in all solutions to block Kv3 currents (see methods). Inset: representative trances are shown from one cell at +13mV.

Figure 4. Kv2.2 mRNA and protein are present in MNTB principal neurons
A. Qrt-PCR of Kv3.1, 2.1 & 2.2 mRNA, expressed relative to Kv3.1. Each value is the mean of 3 separate reactions normalized to actin transcripts. B. Western blots with the Kv2.2 antibody in brain tissue from CBA mice (WT, centre right) and following pre-incubation with the blocking peptide (BP) shows a band at around 110kD. The left blots using the same Kv2.2 antibody in brain tissue obtained from homozygous Kv2.2
knockout mice (Lexicon, TF1551) and control littermates WT (centre left). C. Kv2.2
immunoreactivity (green) is present in the MNTB and VNTB of the superior olivary
complex and at lower levels across the auditory brainstem. Bright ‘sparks’ of
fluorescence can be seen in the MNTB. D. At higher magnification MNTB principal
neurons and the fluorescent sparks are clear. A plot of intensity along the dashed white
line shows the incidence of the highly fluorescent sparks (red dashed lines) often
alongside a neuronal profile (*). Inset (top) shows blocking peptide negative control. E.
A confocal projection of one MNTB neuron double labeled for Kv2.2 and DAPI (blue
nucleus) showing a highly stained putative initial segment region (arrow). F. A confocal
projection of a triple labeled MNTB neuron showing co-localization of Kv2.2 (green) with
Kv1.2 (red) in adjacent parts of the axon initial segment (AIS) and DAPI (blue).

**Figure 5.** A HH model of an MNTB neuron shows that Kv2 regulates the inter-
spike potential and affects availability of Nav channels during high-frequency
firing.

A. Time constants used to generate the model Kv2.2 were obtained from a fit (dashed
line) to the activation and deactivation time constants obtained from voltage clamp
experiments (see Figure 2). B. The magnitudes of the ionic conductances present in the
implementation of the single compartment MNTB model adapted from Macica et al.
2003. C. The membrane potential of the model MNTB neuron in response to synaptic
trains at 50Hz (left) and 200Hz (right) in the presence (black) and absence (grey) of the
Kv2.2 conductance; arrows indicate AP peak and inter-spike potential at the end of each
train, respectively. D. The magnitude of the Kv2.2 current passing during the APs in the
traces shown above in part C. Note that the Kv2.2 current dramatically increases in
magnitude at the start of the 200Hz train (right) but is smaller and relatively stable in the
50Hz train (left). Note the difference in inter-spike potential (black arrow) and action potential height (grey arrow).

**Figure 6. Frequency-dependent hyperpolarization of the inter-spike potential under physiological conditions.** A. Example 50 Hz (black) and 200 Hz (grey) trains of APs in the same MNTB neuron evoked with identical current trains at the two frequencies. Note the different time-scales. Dashed lines indicate the peak of the 1st inter-spike potential and arrows show the peak of the last. Note that AP amplitudes remain fairly constant throughout, but the hyperpolarization of the inter-spike interval is greater with higher frequency stimulation. The 1st and 60th action potentials from 50 and 200 Hz overlain (black & grey respectively) shows that only the inter-spike voltage changes C. Plot of the peak inter-spike potential for the cell in part A.

**Figure 7. Sensitivity of $I_{Na}$ to the inter-spike potential**

A. The steady state inactivation of $I_{Na}$ was determined by plotting the peak current measured at -4mV against the voltage of the 500ms pre-pulse, which was fit with a Boltzmann distribution giving a $V_{1/2in}$ of -55.4±1.7mV and a $k_{in}$ of 6.3±0.1 (n=5), inset shows example traces. B. The protocol used to asses the sensitivity of $I_{Na}$ to changes in inter-spike potential ($V_{is}$); a 500ms -94mV pre-pulse (to remove all steady state inactivation) was followed by test pulses to -4mV interleaved with 5ms intervals, simulating the time between spikes in a 200Hz train, as indicated by the overlaid AP (red). C. Example traces (all from one cell) generated from the protocol in B, show the sensitivity of $I_{Na}$ to $V_{is}$. Black, $V_{is}$ hyperpolarized from -55 to -59 mV (identical to Figure 5D). Blue, $V_{is}$ is kept constant at -55 mV. Green, $V_{is}$ depolarized from -55mV to -51 mV; mimicking mild summation of the inter-spike potential. D. Average $I_{Na}$ amplitudes during
the trains shown in C; data normalised to 2\textsuperscript{nd} spike to show change from the initial level of inactivation. Asterisks indicate onset of statistical significance.

**Figure 8. Age and tonotopic gradients of Kv2.2 current.**

A. The current measured 40ms into a +13mV step depolarization (peak activation) is plotted against postnatal age. A significant increase in the current magnitude is observed with development (statistical significance assessed by ANOVA P<0.001). B. The MNTB nucleus was divided into 3 parts, medial (M), intermediate (I) and lateral (L). C. The current measured 40ms into a +13mV step depolarization is plotted against location for P12 animals. A significant trend to larger currents in lateral neurons was seen across the tonotopic axis. N.B. all data recorded in the presence of 10nM DTx and 3mM TEA.

**Supplementary Figure S1: Functional Kv2.2 is restricted to the axon; evidence that Kv1.2 and Kv2.2 channels share similar or adjacent compartments.**

A. An example of an I/V from a single neuron showing the effect of blocking Kv1 channels with DTx-I (10 nM), which are known to be in the AIS. Note the larger proportional block observed at negative voltages (black arrow) compared to positive voltages (purple arrow, n=6). B. The phenomena shown in (A) causes a distortion of the Kv1 activation curve (blue trace) with the largest change coinciding with the Kv2 activation curve (positive to the dashed line). A plausible explanation for this phenomenon is that since functional Kv1 channels predominate in the AIS (Dodson et al, 2002), block of Kv1 conductance increases both the local input resistance and axon length constant. This gives better voltage control over other conductances in the axon (i.e. Kv2) and therefore results in measurement of a larger Kv2 current by the patch pipette in the somatic compartment. Conversely, without block of Kv1 they shunt this local compartment and attenuate voltage command and measured current magnitudes.
(effectively preventing the local axonal compartment from reaching the command voltage). When Kv1 channels are blocked (with DTx-I) voltage control of the axon improves, higher voltages are now achieved, so recruiting more of axonal high-voltage-activated current. Hence the non-linearity reflects more measured Kv2.2 current in the absence of the Kv1 shunt.
Figure 1
Figure 2

**Activation**

- Voltage Dependence of Activation
  - \( y = 8.2e^{-x/17.7} + 2.3 \)

**Deactivation**

- Voltage Dependence of Deactivation
  - \( y = 24.7e^{x/30.6} + 3.2 \)
Figure 3

A

B

C

[Diagram A showing voltage-current relationship with TEA & DTx, 2 nA 25 ms, +100μM Quin, GHK fit, 100 ms.

Diagram B showing voltage-current relationship with TEA & DTx, 2 nA 25 ms, +5 mM 4-AP.

Diagram C showing Pocc as a function of [TEA] (mM) with IC50 = 11.8.
Figure 5

A. Activation and Deactivation

- Solid line: Activation and Deactivation
- Dashed line: Fit

B. Conductance

<table>
<thead>
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<tr>
<td>Leak</td>
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<tr>
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<tr>
<td>Fast Syn</td>
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</tbody>
</table>

C. Model: 50 Hz

- Black: With Kv2.2
- Grey: Without Kv2.2

D. Model: 200 Hz

- Black: With Kv2.2
- Grey: Without Kv2.2
Figure 6

(A) Graphs showing electrical activity at 50 Hz and 200 Hz.

(B) Comparison of first and 60th action potentials (APs) at 50 Hz and 200 Hz.

(C) Graph depicting peak inter-spike potential with voltage (mV) on the y-axis and spike number on the x-axis, showing differences between 50 Hz and 200 Hz.
Figure 8

A

Current at +13mV (nA) vs. Postnatal day

R²=0.3413
P=<0.0001

B

Midline

C

Current at +13mV (nA)

R²=0.158
P=0.0346
Supplemental Figure S1